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Effects of Assembly and Mutations Outside the Active Site on the Functional pH Dependence of *Escherichia coli* Aspartate Transcarbamylase*

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Electrostatics are central to the function and regulation of *Escherichia coli* aspartate transcarbamylase, and modeling has suggested that long range electrostatic effects are likely to be important (Glackin, M. P., McCarthy, M. P., Mallikarachchi, D., Matthew, J. B., and Allewell, N. M. (1989) *Proteins Struct. Funct. Genet.* 5, 66–77; Oberoi, H., Trikha, J., Yuan, X., and Allewell, N. M. (1995) *Proteins Struct. Funct. Genet.*, in press). To investigate this possibility from an experimental standpoint, we have examined the effects both of assembly and of removing ionizable and polar side chains outside the active site (Glu-50, Tyr-165, and Tyr-240) on the pH dependence of the kinetic parameters of aspartate transcarbamylase. The holoenzyme (c_6r_6) assembles from three regulatory dimers (r_2) and two catalytically active trimers (c_3). pH dependences of the enzyme kinetic parameters suggest that the mechanisms of productive binding of L-Asp to the binary complexes of the catalytic subunit (c_3) and holoenzyme (c_6r_6) with carbamyl phosphate are different. In contrast, the Michaelis complex appears similar for both c_3 and c_6r_6, except for p*K* shifts of ~1 pH unit. Results also indicate that the catalytic mechanism of the holoenzyme does not involve reverse protonation, as has recently been proposed for the catalytic trimer (Turnbull, J. L., Waldrop, G. L., and Schachman, H. K. (1992) *Biochemistry* 31, 6562–6569). The tyrosines at positions 165 and 240 are part of a cluster of interactions that links the catalytic subunits in the T state (the c1:c4 interface) and which is disrupted in the T → R transition. The effects of mutating the two Tyr residues are quite different: Y240F has higher than wild-type activity and affinity over the entire pH range, while Y165F has activity and affinity an order of magnitude lower than wild-type. Removal of the regulatory subunits from Y165F increases activity and affinity and restores the pH dependence of the wild-type catalytic subunit. Like Y165F, E50A has low activity and affinity over the entire pH range. Linkage analysis indicates that there is long range energetic coupling among the active site, the c:r subunit interfaces, and residue Y165. The substantial quantitative difference between Y165F and Y240F, both of which are at the c1:c4 interface about 14–16 Å from the closest active site, demonstrates specific path dependence, as opposed to general distance dependence, of interactions between this interface and the active site.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) was one of the first systems exploited to study intramolecular signal transduction (Yates and Pardee, 1956) and remains an important system for analyzing molecular mechanisms of recognition, communication, and regulation. This enzyme catalyzes the first committed step in pyrimidine biosynthesis, formation of *N*-carbamyl-L-Asp from carbamyl phosphate and L-aspartate. Aspartate transcarbamylase binds L-Asp cooperatively, and its enzymatic activity is allosterically regulated by nucleotide triphosphates (Yates and Pardee, 1956; Gerhart and Pardee, 1962, 1963; Wild *et al.*, 1989). Recent reviews include Allewell (1989), Hervé (1989), Kantrowitz and Lipscomb (1990), Wild and Wales (1990), and Lipscomb (1992, 1994).

The protein is a dodecamer consisting of six catalytic (c)[1] chains and six regulatory (r) chains organized as two catalytic trimers (c_3) and three regulatory dimers (r_2). Binding of substrates and substrate analogs induces a T → R transition in which the holoenzyme expands by 12 Å along its 3-fold axis. The switch to the high affinity R structure eliminates contacts between c_3 subunits (the C1:C4 interface), one set of c_3-r_2 contacts and domain interactions in the r chain but simultaneously strengthens a second set of c_3-r_2 contacts, interchain interactions in c_3 and interdomain interactions in c chains. Critical features of this transition include large movements of the 80s and 240s loops in the c chains and closure of the c chain domains (see Fig. 1).

Because both the substrates of aspartate transcarbamylase and its regulatory nucleotides have several negative charges, electrostatic effects might be expected to figure heavily in both catalysis and the allosteric mechanism. A number of studies have substantiated this expectation. Gerhart and Pardee (1964) showed that the pH optimum for catalytic activity shifts from 7 at low aspartate concentrations to 8.3 at high aspartate concentrations. These pH optima are assigned to the T and R structural states of the enzyme. In the first systematic study of pH effects, Pastra-Landis *et al.* (1978) demonstrated differences in the pH dependence of the holoenzyme and catalytic subunit. Thiry and Hervé (1978) and Tauc *et al.* (1982) showed that the effects of ATP and CTP on enzymatic activity vary markedly with pH. Mutants for which the pH dependence of catalytic activity is different from the wild-type have been found (Ladjimi and Kantrowitz, 1987; Xi *et al.*, 1990; Waldrop

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This paper is dedicated to the memory of Dr. Fred Wedler, who was a highly creative and careful kineticist and a very warm and generous person.

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[1] The abbreviations used are: c, catalytic chain; r, regulatory chain; PALA, *N*-[phosphonoacetyl]-L-aspartate; bis-Tris, 2-[bis(2-hydroxyethy)amino]-2-(hydroxymethyl)-propane-1,3-diol; Caps, 3-(cyclohexylamino)propanesulfonic acid.

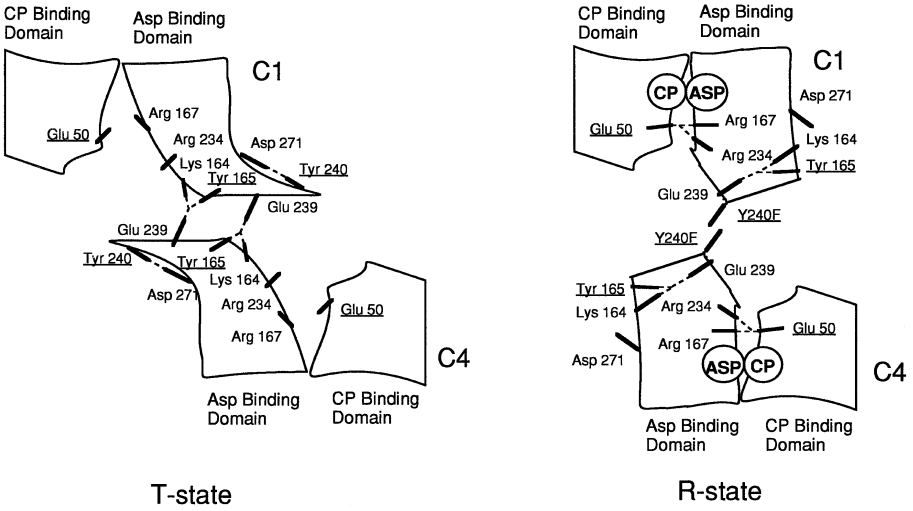


FIG. 1. **Schematic diagram showing the interactions of residues Glu-50, Tyr-165, and Tyr-240 in the unligated (T state) and ligated (R state) forms of the enzyme.** One of the three symmetric c1:c4 type interfaces in the holoenzyme is shown (the equivalent interfaces in the holoenzyme are c2:c5 and c3:c6). Each c chain is comprised of an aspartate and a carbamyl phosphate binding domain. The c1:c4 interface in the T state consists of symmetric interactions between the 240s loop of each chain and residues 164 and 165. There is no c1:c4 interface in the R state: here the 240s loop of each chain interacts with residues 164 and 165 of that same chain. This figure is adapted from a figure by Ladjimi and Kantrowitz (1988) and is based on the crystal structures solved in the Lipsomb laboratory (Honzatko *et al.*, 1982; Ke *et al.*, 1984; Krause *et al.*, 1985, 1987).

et al., 1992a, 1992b). pK values of groups involved in binding and/or catalysis have been derived from the pH dependence of the kinetic parameters of c_3 (Leger and Hervé, 1988; Turnbull *et al.*, 1992), and a model involving three active site residues has been proposed (Turnbull *et al.*, 1992). Allewell *et al.* (1975) showed that nucleoside triphosphate binding is linked to proton binding, and in a series of subsequent papers they defined linkages between proton binding and binding of substrates, competitive inhibitors, and nucleotides and the assembly reaction (Knier and Allewell, 1978; Allewell *et al.*, 1979; Burz and Allewell, 1982; McCarthy and Allewell, 1983). Computer modeling indicates that these linkages result from large changes in the pK_a values of groups widely distributed throughout the molecule (Glackin *et al.*, 1989, 1991).[2]

In this study we have examined the pH dependence of the kinetics of the holoenzyme and a set of single site mutants in which ionizable or polar side chains outside the active site have been replaced by nonionizable, nonpolar side chains. Two of the mutations (Y165F and Y240F) occur in a cluster of interactions at the c1:c4 interface that forms a critical link between catalytic subunits in the T structural state and is disrupted in the T → R transition (Fig. 1). E50A is an interdomain mutant (in the carbamyl phosphate-aspartate domain interface in the c chain) that forms salt bridges with Arg-167 and Arg-234 in the R state (Newton and Kantrowitz, 1990). Because the interactions of these sites differ in the T and R states, there has been interest in determining how mutating them alters the T → R equilibrium. The conclusion varies depending upon whether the structure of the protein or its function is examined. On the basis of substrate affinity and catalytic efficiency at a single pH, along with other diagnostics such as their response to PALA (*N*-[phosphonoacetyl]-L-aspartate), CTP, and ATP or sulfhydryl reactivity, two of these mutants (Y165F, E50A) appear to be shifted toward the functional T state (Wales *et al.*, 1988; Newton and Kantrowitz, 1990). Functionally, Y240F appears to be shifted toward the R state (Ladjimi and Kantrowitz, 1988; Middleton and Kantrowitz, 1986; Middleton *et al.*, 1989); however, the crystal structure of Y240F in the absence of ligands has a T state quaternary structure, except in the vicinity of Tyr-240 (Gouaux *et al.*, 1989).

Examination of the function of these mutants over a wide pH range confirms some earlier conclusions based on results at a single pH and allows us to examine previously inaccessible elements of the role of electrostatics in the molecular mechanism of aspartate transcarbamylase, including 1) the effect of assembly on the ionization behavior of residues involved in binding and catalysis and 2) the non-distance-dependent involvement of long range electrostatic effects in catalysis and regulation of the holoenzyme. The present data also allow us to advance the understanding of several other questions. 1) Can pH drive the T → R equilibrium? 2) Can models involving a limited number of ionizable groups be developed to account for the pH dependence of the kinetic parameters of the aspartate transcarbamylase holoenzyme and the effects of mutations? 3) What is the energetic coupling between the mutated residues and ionizable groups involved in the catalytic mechanism?

MATERIALS AND METHODS

Proteins—Wild-type and mutant holoenzymes were isolated from *E. coli* strain EK1104 transformed with the appropriate plasmid as described previously (Nowlan and Kantrowitz, 1985). EK1104 and plasmids containing the wild-type pyrB gene (pEK2), and Y240F (pEK33) were provided by Dr. E. R. Kantrowitz (Department of Chemistry, Boston College, Chestnut Hill, MA 02167). Plasmid containing the mutant gene for Y165F was obtained from Dr. J. R. Wild (Department of Biochemistry and Biophysics, Texas A & M University, College Station, TX). E50A was obtained as the purified protein from Dr. Kantrowitz.

c_3 subunits were prepared following Yang *et al.* (1978) with the modifications introduced by Burz and Allewell (1982). Proteins were stored as precipitates in 3.6 M $(NH_4)_2SO_4$, 0.1 M Tris-HCl, 0.2 mM EDTA, and 0.2 mM dithiothreitol, pH 8.3, at 4 °C. The purity of c_6r_6, assessed by nondenaturing PAGE (Davis, 1964) at pH 8.3, was found to be greater than 95%, with only c_6r_4, c_3, and aggregates being detectable as contaminants.

Protein concentrations for wild-type and E50A were determined spectrophotometrically at 280 nm using extinction coefficients of 0.59 ml·(mg·cm)$^{-1}$ for c_6r_6 and 0.72 ml·(mg·cm)$^{-1}$ for c_3 (Gerhart and Holoubek, 1967). Extinction coefficients for Y165F and Y240F were determined using a colorimetric protein concentration assay (Bio-Rad Protein assay, Bio-Rad); the values obtained were 0.56 and 0.52 ml·(mg·cm)$^{-1}$, respectively.

Chemicals—Carbamyl phosphate, L-Asp, carbamyl L-Asp, Tris, bis-Tris, and Caps were purchased from Sigma. The dilithium salt of carbamyl phosphate was recrystallized from ice-cold 50% ethanol and stored desiccated at −20 °C (Gerhart and Pardee, 1962). PALA, the bisubstrate analog, assayed as >80% pure and used without further purification, was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892. Neohydrin (1-[3-(chloromercuri-2-methoxypropyl)urea]) was a gift from Marion Merrell Dow Pharmaceutical Inc. (Cincinnati, OH) and was recrystallized from absolute ethanol before use.

Assay—The colorimetric assay, developed by Prescott and Jones (1969) and modified by Pastra-Landis *et al.* (1978), was used to monitor formation of carbamyl-L-Asp. The buffer system used was 0.02 M Tris-Cl, 0.02 M bis-Tris, and 0.02 M Caps, 0.2 mM dithiothreitol, 0.2 mM

[2] H. Oberoi and N. M. Allewell, manuscript in preparation.

EDTA (Pastra-Landis *et al.*, 1978). Assays were carried out at 25 °C, and assay tubes were equilibrated in a water bath for at least a half of an hour before each assay. The amount of enzyme used in each experiment, which varied with pH and the mutant being studied, was chosen to produce 0.02–0.2 μmol of carbamyl L-Asp in 5 min. Separate carbamyl-L-Asp standard curves were generated for each individual titration curve, since this assay is highly sensitive to room temperature, ambient light, and the batch of color reagent. Stock carbamyl phosphate solutions (48 mM) were prepared fresh in buffer immediately before each titration and kept on ice to keep decomposition to a minimum.

Data Analysis—The nonlinear least squares program NONLIN (Johnson and Frasier, 1985) was used throughout. We initially attempted to resolve the kinetic parameters using the approach of Pastra-Landis *et al.* (1978). In the absence of substrate inhibition, saturation curves were fit to the Hill equation

$$V = \frac{V_{max}[S]^{n_H}}{K' + [S]^{n_H}} \quad \text{(Eq. 1)}$$

where $K' = (K_m^{app})^{n_H} \cong [S]_{0.5}^{n_H}$. In the presence of substrate inhibition, the data were initially fit to the equation

$$V = \frac{V_{max} + V_i[S]/K_i}{1 + K'/[S]^{n_H} + [S]/K_i} \quad \text{(Eq. 2)}$$

where K_i is the dissociation constant for inhibitory substrate binding and V_i is the limiting velocity when substrate inhibition is maximal (Pastra-Landis *et al.*, 1978). However, values of the fitted parameters obtained with this equation were highly correlated and the fitted values of V_{max} and K_m^{app} were much larger than appeared reasonable in light of the raw data, as Pastra-Landis *et al.* (1978) reported. We find that the equation

$$V = \frac{V_{max} + V_i[S]^2/K_i^2}{1 + K'/[S]^{n_H} + [S]^2/K_i^2} \quad \text{(Eq. 3)}$$

eliminates these problems.[3] This equation describes cooperative substrate binding, incorporated as a Hill coefficient, and partial uncompetitive substrate inhibition.

Plots of log V_{max} and log V_{max}/K_m^{app} *versus* pH were fit to equations in which these parameters are partitioned into contributions from molecules in different states of ionization and with different catalytic efficiencies and/or substrate affinities (Cleland, 1977; Turnbull *et al.*, 1992). With the exception of Y165F, all of the data could be fit by models that assume that both V_{max} and V_{max}/K_m^{app} are modulated by the ionization states of one or two independent groups

$$\begin{array}{ccccc} & & K_m{}^{a} & & k_{cat}{}^{a} \\ E\mathrm{H_2\text{-}CP} + \mathrm{Asp} & & \rightharpoonup & E\mathrm{H_2\text{-}CP\text{-}Asp} & \rightarrow E\mathrm{H_2} + P \\ \mathrm{p}K_1' \uparrow\downarrow & & K_m{}^{b} \ \mathrm{p}K_1 \uparrow\downarrow & & k_{cat}{}^{b} \\ E\mathrm{H\text{-}CP} + \mathrm{Asp} & & \rightharpoonup & E\mathrm{H\text{-}CP\text{-}Asp} & \rightarrow E\mathrm{H} + P \\ \mathrm{p}K_2' \uparrow\downarrow & & K_m{}^{c} \ \mathrm{p}K_2 \uparrow\downarrow & & k_{cat}{}^{c} \\ E\text{-CP} + \mathrm{Asp} & & \rightharpoonup & E\text{-CP-Asp} & \rightarrow E + P \end{array}$$

SCHEME I.

where E is the free enzyme, E-CP is the binary complex of aspartate transcarbamylase with carbamyl phosphate, and E-CP-Asp is the ternary complex of aspartate transcarbamylase with carbamyl phosphate and L-Asp. Following Cleland (1977), the equation for a single ionizable group is

$$\log Y = \log((Y_a + Y_b(K_a/H))/(1 + K_a/H)) \quad \text{(Eq. 4)}$$

where Y is either V_{max} or V_{max}/K_m^{app}, Y_a and Y_b are the fitted values of the appropriate parameter for the appropriate protonated and unprotonated species, and K_a is the appropriate microscopic dissociation constant (K_1 for V_{max} plots; K'_1 in V_{max}/K_m plots). The general equation for the ionization of two independent groups is

$$\log Y = \log((Y_a(H/K_a) + Y_b + Y_c(K_b/H))/(1 + H/K_a + K_b/H)) \quad \text{(Eq. 5)}$$

where K_a and K_b are macroscopic acid dissociation constants, which are equal to the microscopic constants K_1 and K_2 when $\mathrm{p}K_b - \mathrm{p}K_a > 1.5$. When $\mathrm{p}K_b - \mathrm{p}K_a < 1.5$, $K_a = K_1 + K_2$ and $K_aK_b = K_1K_2$. As in Equation 4, Y_a, Y_b, and Y_c are the fitted values of V_{max} or V_{max}/K_m^{app} for different ionization states of the protein. The higher the index, the lower the protonation state of the protein. Since plots of both log V_{max} and log V_{max}/K_m^{app} had two maxima for Y165F, these curves were fit to Equation 6, which assumes that ionization in the low pH region does not affect ionization in the high pH region.

$$\log Y = \log[Y_{b1}/(1 + K_a/\mathrm{H} + \mathrm{H}/K_b) + Y_{b2}/(1 + K_c/\mathrm{H} + \mathrm{H}/K_d)] \quad \text{(Eq. 6)}$$

For simplicity, Y_{a1}, Y_{c1}, Y_{a2}, and Y_{c2} were assumed to have values of zero, such that Y_{b1} and Y_{b2} correspond to the singly protonated low and high pH forms.

Correlation of the "group" pK_a values resolved from such analyses with specific ionizable residues in the enzyme active site is a complex and often assumption-fraught exercise (Knowles, 1976; Brocklehurst, 1994). It minimally requires that one demonstrate or assume that each apparent pK_a corresponds to a single side chain and not the net sum of several overlapping ionizations, that these side chains are in the active site itself, that for each activity maximum in the pH dependence there is only one ionization state of the active site that is enzymatically active, and that the kinetic mechanism of the enzyme does not change appreciably with pH. As this study demonstrates, however, comparative analysis of the quantitative pH dependence of different mutants and states of assembly of the enyzme does allow one to examine many aspects of the involvement of electrostatics in the mechanism of aspartate transcarbamylase without specific side chain assignments.

RESULTS

Aspartate Titration Curves—Selected sets of data illustrating the effects of pH on L-Asp saturation curves are shown in Fig. 2. The effects of the mutations on both activity and affinity are much greater than the effects of pH, *i.e.* while both the K_m^{app} and V_{max} of the low activity mutants (E50A and Y165F) span a 4–5-fold range dependent on pH, they both differ by an order of magnitude in their affinity and activity from the wild-type and Y240F enzymes (see also Fig. 3). The pronounced substrate inhibition seen at pH 8.3 and higher in the wild-type enzyme and Y240F is a striking feature of these data. Substrate inhibition either does not occur in the low activity mutants or occurs only at aspartate concentrations greater than 350–400 mM.

The effects of mutating the two Tyr residues in the c1:c4 interface are quite different, with Y240F increasing affinity for L-Asp and catalytic activity above the levels seen in the wild-type enzyme and Y165F having greatly reduced affinity and activity relative to the native enzyme. Removing the r_2 subunits of Y165F largely restores its catalytic activity and partially restores L-Asp affinity (see also Fig. 5). E50A, the interdomain mutant, shows reduced affinity and activity, similar to Y165F. The effects of removing the r_2 subunits of E50A were not examined.

pH Dependence of K_m^{app}, V_{max}, and n_H—The values of the kinetic parameters derived by fitting the titration curves of the wild-type enzyme and the mutants to Equations 1 or 3 are plotted as a function of pH in Fig. 3. The kinetic properties of the wild-type enzyme and Y240F are very similar over the entire pH range, although the maximal velocity and substrate affinity of Y240F are larger by factors of 1.5 and 2, respectively, than the values for the wild-type enzyme, while its Hill coefficient never exceeds a value of 1.7. Both wild-type and Y240F show maximal values for V_{max} at pH 9.5 and for K_m^{app} at pH 9.0.

Y165F and E50A both display low activity and affinity over the entire pH range. Although the pH dependence of n_H of E50A is similar to the wild-type enzyme, the maximum n_H for Y165F, as well as the largest values of V_{max} and K_m^{app} for both E50A and Y165F are at or near pH 7 (the K_m^{app} maximum is at pH 7.5 for Y165F). pH 7 is commonly considered to be the functional pH optimum for the T form of aspartate transcarbamylase, based on measurements of the pH dependence of velocity at single aspartate concentrations (see "Discussion"). Although the low activity and affinity of Y165F and a value of ~1 for n_H above pH 7.5 suggests that its ability to undergo the

[3] V. J. LiCata and N. M. Allewell, manuscript in preparation.

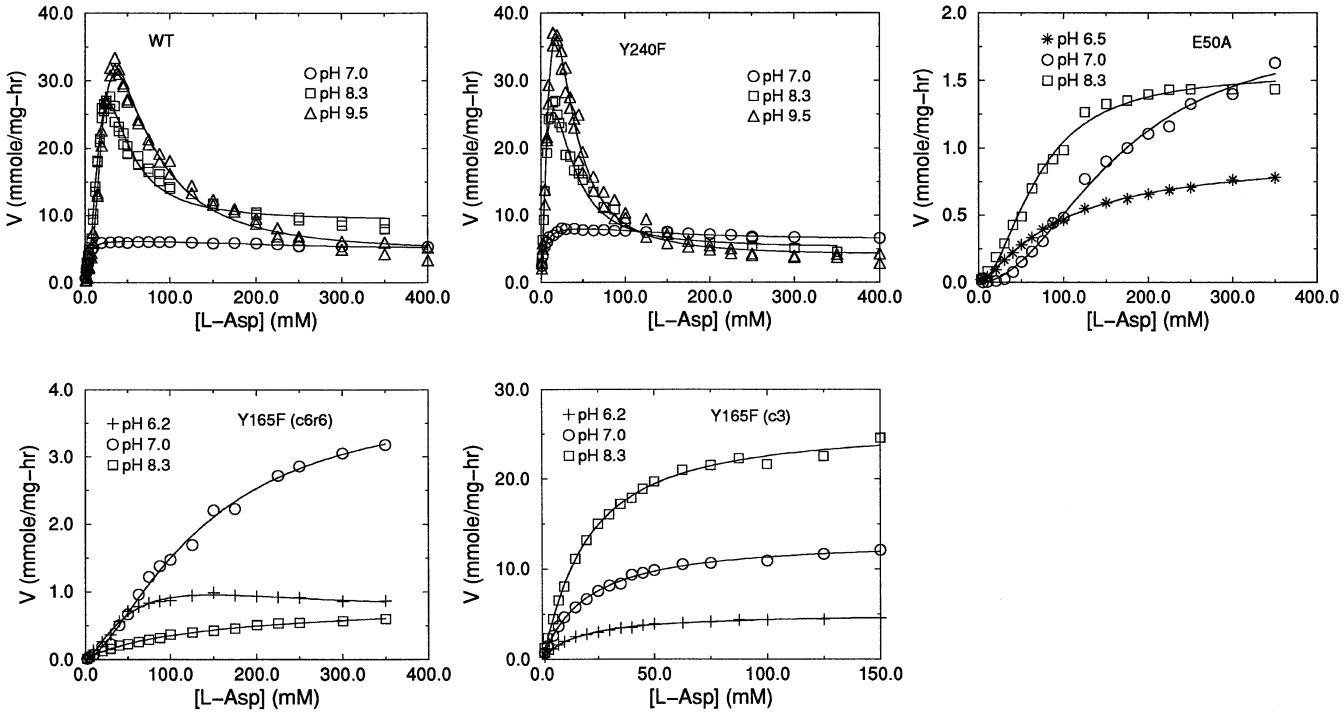


FIG. 2. **pH dependence of [L-Asp] titration curves.** Although 10–15 titration curves were obtained for the wild-type, Y240F, E50A, and Y165F enzymes, for clarity only three curves are shown. *Solid lines* show the fits of the data to Equations 1 or 3 as described in the text. All experiments were carried out in 0.02 M Tris-Cl, 0.02 M bis-Tris, 0.02 M Caps, 0.2 mM dithiothreitol, 0.2 mM EDTA at 25 °C in the presence of 4.8 mM carbamyl phosphate.

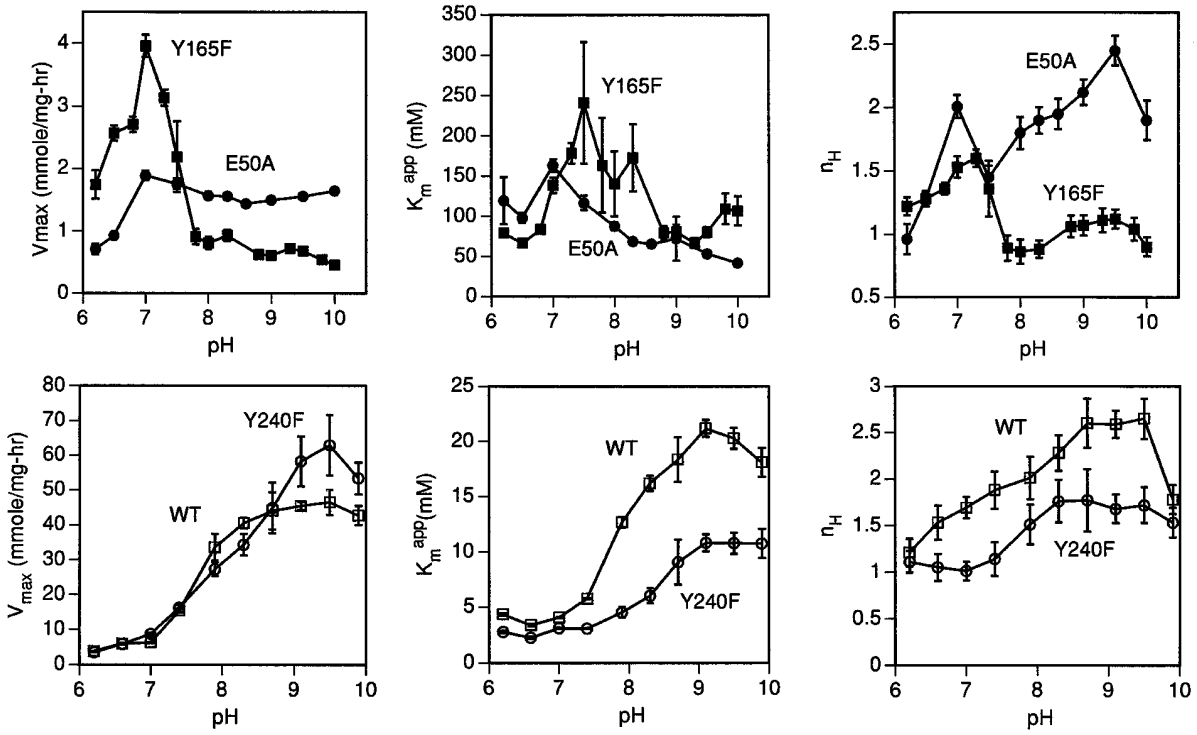


FIG. 3. **pH dependence of the kinetic parameters of wild-type and mutant enzymes.** The V_{max}, K_m^{app}, and Hill coefficient (n_H) for each enzyme are shown as a function of pH. *Upper panels* show the low activity mutants Y165F (■) and E50A (●). *Lower panels* show the wild-type enzyme (□) and high activity mutant Y240F (○).

T → R transition may be compromised, it is still activated by PALA by a factor of 17 at pH 8.3 (data not shown; see also Wales *et al.* (1988)). Likewise, E50A is activated nearly 6-fold by PALA (data not shown).

The low substrate affinity and catalytic activity of Y165F and the unusual pH dependence of these parameters must depend upon subunit interactions in the holoenzyme. When the regulatory subunits are removed, both substrate affinity and V_{max} increase by approximately a factor of six, and the pH dependence of these parameters becomes similar to those of wild-type

TABLE I
Fitted pK_a values

a. Fits to a single pK_a value

	log V_{max} (pK_1)	log V_{max}/K_m^{app} (pK'_1)
WT (c_6r_6)	7.84 ± 0.11	6.40 ± 0.13
Y240F (c_6r_6)	7.91 ± 0.11	6.73 ± 0.13
E50A (c_6r_6)	6.19 ± 0.14	6.82 ± 0.16

b. Fits to two pK_a values

	log V_{max}		log V_{max}/K_m^{app}	
	pK_1	pK_2	pK'_1	pK'_2
WT (c_6r_6)	7.88 ± 0.07	10.49 ± 0.49	6.68 ± 0.07	7.84 ± 0.14
WT (c_3)[a]	7.16 ± 0.11	9.51 ± 0.09	7.03 ± 0.14	9.13 ± 0.10
Y240F (c_6r_6)	7.96 ± 0.07	10.92 ± 0.31	7.08 ± 0.20	7.86 ± 0.27
E50A (c_6r_6)	6.73 ± 0.10	7.18 ± 0.37	6.95 ± 0.15	9.38 ± 0.32
Y165F (c_3)	6.88 ± 0.09	9.48 ± 0.08	6.90 ± 0.23	8.84 ± 0.17

c. Fits to four pK_a values

	log V_{max}			
	pK_1	pK_2	pK_3	pK_4
Y165 (c_6r_6)	6.46 ± 0.18	7.32 ± 0.08	9.04 ± 0.20	9.76 ± 0.17
	log V_{max}/K_m^{app}			
	pK'_1	pK'_2	pK'_3	pK'_4
Y165F (c_6r_6)	5.81 ± 0.42	6.34 ± 0.25	8.84 ± 0.18	9.65 ± 0.22

[a] From Turnbull *et al.* (1992), in 0.1 M MES, 0.051 M *N*-ethylmorpholine, and 0.051 M diethanolamine with 0.2 mM EDTA and 2 mM β-mercaptoethanol.

c_3, with maxima for V_{max} and K_m^{app} at 8.5 and 9.5, respectively (see Fig. 5 and Table I).

Substrate Inhibition—As shown in Fig. 4, values of log K_i derived by fitting to Equation 3 have a reverse bell-shaped dependence on pH. Substrate inhibition is strongest between pH 8 and 9, where the wild-type enzyme and Y240F are most catalytically active but substrate affinity is lowest (compare with Fig. 5). pK values for substrate inhibition derived by fitting to Equation 5 are 7.8 ± 0.2 and 9.5 ± 0.2 for the wild-type enzyme and 7.5 ± 0.2 and 10.0 ± 0.2 for Y240F.

Apparent pK Values—pK values of ionizable groups involved in substrate binding and catalysis are often derived by examining the pH dependence of log V_{max}/K_m^{app} and log V_{max} (Cleland, 1977; Dixon *et al.*, 1979). The first type of plot identifies groups involved in binding and/or catalysis; the second identifies groups involved only in catalysis. This approach has been applied to c_3 by Leger and Hervé (1988) and Turnbull *et al.* (1992). When this approach is applied to the holoenzyme, interpretation becomes more complicated, since the individual pK_a values resolved by this analysis may reflect the aggregate behavior of several titrating groups and/or the behavior of groups outside the active site, particularly those involved in assembly and the T → R transition.

Plots of log V_{max} and log V_{max}/K_m^{app} *versus* pH for wild-type, Y240F, Y165F, and E50A holoenzymes and Y165F c_3 are shown in Fig. 5. Plots of log V_{max} for wild-type enzyme, Y240F, and E50A plateau at high pH, and hence can be fit to one pK_a. In contrast, the double maxima in both Y165F plots indicate that there are at least two active species of this holoenzyme. The log V_{max} and log V_{max}/K_m^{app} profiles for the holoenzymes are obviously different from those of wild-type c_3 (Turnbull *et al.*, 1992) and Y165F c_3.

Although the results for the wild-type, Y240F, and E50A holoenzymes can be fit reasonably well to a single ionizable groups, they were fit to both one and two groups so that these fits could be compared with those derived for c_3 by others. The fitted values of the parameters are given in Tables I and II. Table II reports the fitted Y values as log k_{cat} and log k_{cat}/K_m to facilitate comparison between the different assembly states of the enzyme.

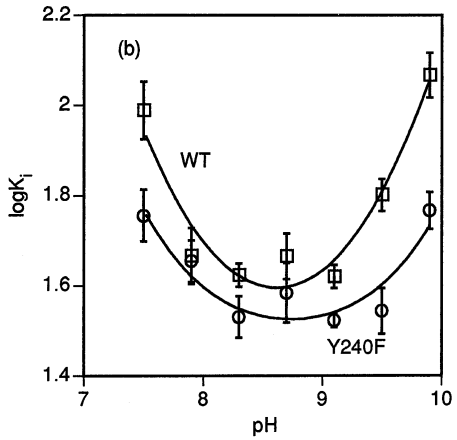


FIG. 4. **pH dependence of log K_i for the wild-type enzyme (□) and Y240F (○).** *Solid lines* are the fits of the data to Equation 5.

Fitting the log V_{max} curves of both wild-type and Y240F holoenzymes to either one or two groups identifies a group with an apparent pK of 7.8–8. The fitted values of k_{cat} for individual species given in Table II indicate that this group is unprotonated in the major active species. Fitting log V_{max}/K_m^{app} of the wild-type and Y240F holoenzymes to one pK_a identifies a group with an apparent pK in the range of 6.4–7.1, while the two group fit identifies a second group with a pK of 7.8. Both fits indicate that in the active species one of these groups is unprotonated (Table II). The major differences between E50A and the wild-type and Y240F holoenzymes are that the second pK is shifted down to 7.2 in the fits to log V_{max} and shifted up to 9.4 in the fits to log V_{max}/K_m^{app}.

Test of the Reverse Protonation Model—Fig. 6 compares the fit to the experimental values of V_{max}/K_m^{app} values for one- and two-group fits in the presence and absence of reverse protonation. Reverse protonation is the participation in the catalytic mechanism of a species in which the ionization state of two groups is the reverse of what would be predicted from their pK values. The model for catalysis in the catalytic trimer proposed by Turnbull *et al.* (1992) postulates reverse protonation of the catalytically productive binary complex with carbamyl phosphate in c_3. The curves in all panels of Fig. 6 are calculated by assuming that only one or two of the several protonation states of the protein is catalytically active (see the legend to Fig. 6). The curves in Fig. 6, *a* and *b*, were calculated assuming there is no reverse protonation (*i.e.* the group with the lower pK ionizes at the lower pH). These curves show that either species X alone (in the one pK_a fits) or species $X + XH_2$ (in the two pK_a fits) as resolved from fitting the log V_{max}/K_m^{app} plots reproduce the original pH dependence of V_{max}/K_m^{app}. In contrast, the curves shown in Fig. 6, *c* and *d*, which were calculated assuming reverse protonation, do not generate the plateau at high pH seen in the wild-type and Y240F data. Note, however, that for the catalytic trimer of mutant Y165F, the reverse protonation model clearly fits the data better.

Thermodynamic Coupling of the Active Site to the 240s Loop and the c:r Assembly Interface—Since changes in pK values correspond to changes in free energies of ionization (ΔG = 2.303 RT pK_a), the change in the pK value of a given group that results from mutating a second group can be thought of as a measure of the interaction energy ($\Delta\Delta G$) between the two groups. By analogy to Loewenthal *et al.* (1993), $\Delta\Delta G_{\text{active site} \leftrightarrow \text{mutation site}} = -RT \ln(K_a^{wt}/K_a^{mutant}) = 1.364\,\Delta pK_a$, where ΔpK_a is the change in the pK_a derived from the pH dependence

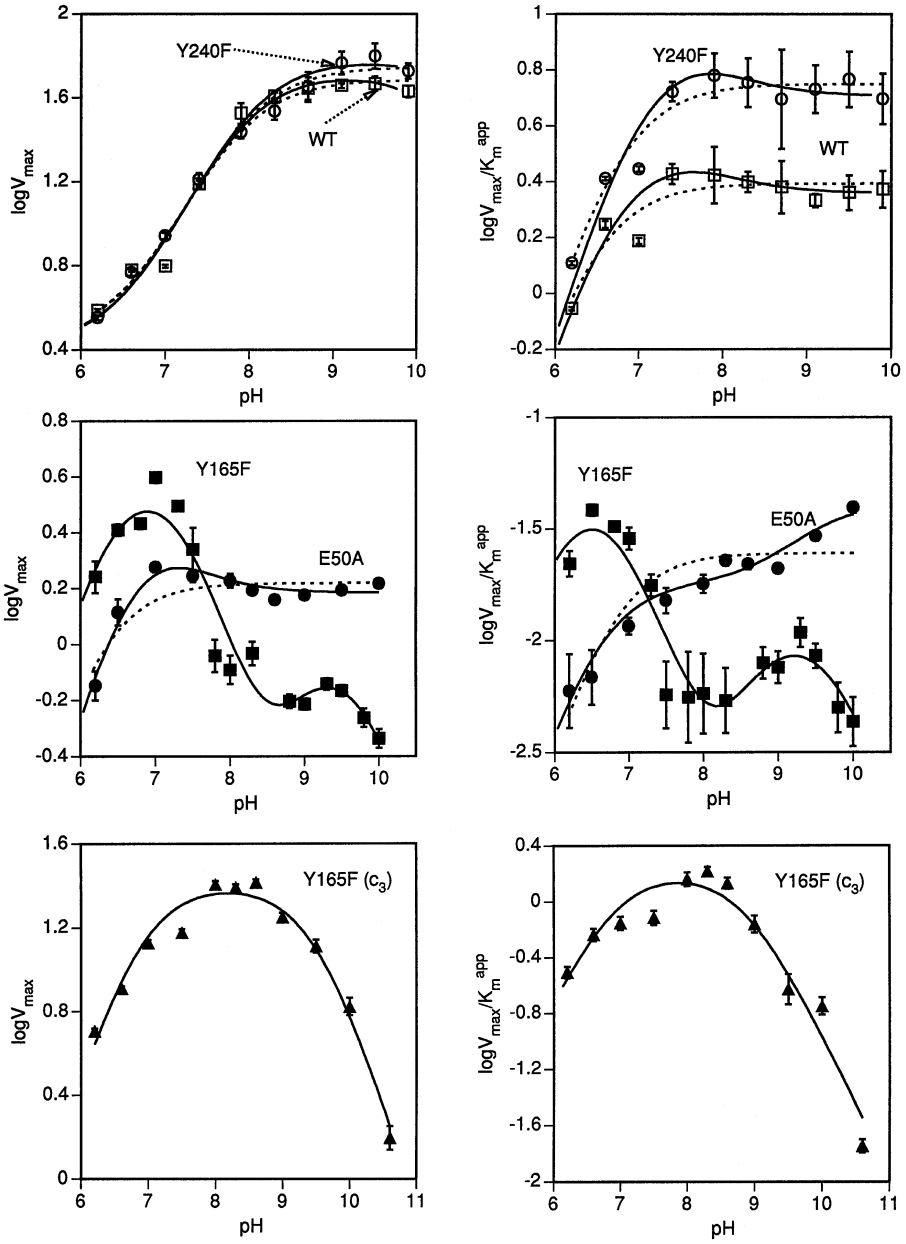
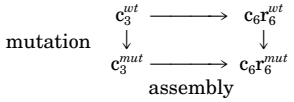


FIG. 5. **pH dependences of log V_{max} and log V_{max}/K_m^{app}**. *Solid lines* are the fits to two pK_a values (Equation 5), or four pK_a values in the case of Y165F (Equation 6). *Dashed lines* are the fits to a single pK_a (Equation 4). Details of the fitting procedure are described under "Materials and Methods." The symbols are experimental points: WT (□), E50A (●), Y240F (○), Y165F c_6r_6 (■), Y165F c_3 (▲). The units for V_{max} and V_{max}/K_m^{app} are mmol·(mg·h)$^{-1}$ and mmol·(mg·h·mM)$^{-1}$, respectively.

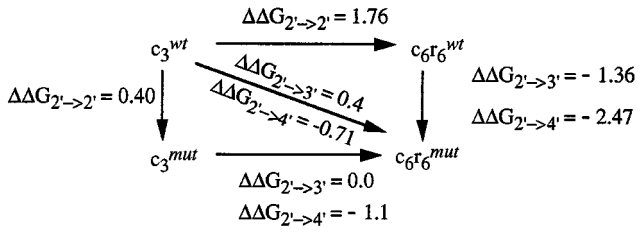
of the wild-type *versus* the mutant enzyme. Similarly, a change in the pK value of a functional group that results from assembling the holoenzyme from its subunits is a measure of the net sum interaction energy between the group and the subunit interfaces. The active site pK shifts caused by assembly and mutation thus reflect long range energetic coupling within the molecule.

The pK values derived for wild-type and mutant catalytic subunits and holoenzymes are linked by the following thermodynamic cycle.



SCHEME II.

The only mutant for which data are available for both c_3 and c_6r_6 is Y165F. Although these data yield values that only crudely approximate real free energies, examination of those thermodynamic cycles that balance provides futher evidence for long range coupling within the molecule. For example, consider the thermodynamic cycles for pK values derived from log V_{max}/K_m plots that link pK'_2 for c_3^{mut}, c_3^{wt}, and $c_6r_6^{wt}$ with pK'_3 or pK'_4 for $c_6r_6^{mut}$.



SCHEME III.

Here the subscripts denote which pK values are being used to calculate the $\Delta\Delta G$ along each pathway, e.g. $\Delta\Delta G_{2'\to 2'}$ along the upper edge of the cycle is the interaction energy estimated from the shift in pK_2 in the wild-type enzyme as it assembles (pK'_2 of $c_3^{wt} \to pK'_2$ of $c_6r_6^{wt}$). All possible linkages among the titrating groups can be examined in this way. Using these linkages, one

TABLE II
Fitted Y values

a. Fits to a single pK_a value

	log k_{cat}[a]		log k_{cat}/K_m^{app}	
	Y_a (XH)	Y_b (X)	Y'_a (XH)	Y'_b (X)
	s^{-1}	s^{-1}	$s^{-1} \cdot mM^{-1}$	$s^{-1} \cdot mM^{-1}$
WT (c_6r_6)	40 ± 8	695 ± 51	0	35 ± 3
Y240F (c_6r_6)	41 ± 8	799 ± 50	0	81 ± 8
E50A (c_6r_6)	0	24 ± 2	0	0.34 ± 0.05

b. Fits to two pK_a values

	log k_{cat}		
	Y_a (H_1XH_2)[b]	Y_b (H_1X + XH_2)	Y_c (X)
	s^{-1}	s^{-1}	s^{-1}
WT (c_6r_6)	36 ± 8	757 ± 54	0
Y240F (c_6r_6)	47 ± 9	871 ± 69	0
E50A (c_6r_6)	0	37 ± 5	22 ± 1
Y165F (c_3)	0	233 ± 23	0
WT (c_3)[c]	0	680 ± 80	0

	log k_{cat}/K_m^{app}			
	Y'_a ($H'_1XH'_2$)	Y'_b (H'_1X + XH'_2)	Y'_c (X)	K_m^{app}[d]
	$s^{-1} \cdot mM^{-1}$	$s^{-1} \cdot mM^{-1}$	$s^{-1} \cdot mM^{-1}$	mM
WT (c_6r_6)	0	47 ± 5	32 ± 4	16.1
Y240F (c_6r_6)	0	112 ± 15	73 ± 11	7.8
E50A (c_6r_6)	0	0.26 ± 0.03	0.59 ± 0.10	142
Y165F (c_3)	0	15 ± 4	0	15.5
WT (c_3)[c]	0	88 ± 10	0	7.8

c. Fits to four pK_a values (Y165F (c_6r_6))

	Y_{b1} ($H_1XH_3H_4$ + $H_2XH_3H_4$)	Y_{b2} (XH_3 + XH_4)
log k_{cat} (s^{-1})	71 ± 7	16 ± 2
log k_{cat}/K_m^{app} ($s^{-1} \cdot mM^{-1}$)	0.95 ± 0.39	0.20 ± 0.037
(K_m^{app})[d] (mM)	74.7	80.4

[a] k_{cat} values are all expressed as turnover/active site, so that c_3 and c_6r_6 can be compared.
[b] The subscript of H corresponds to the pK_a of the ionizable group; for example H_1 is the proton with a pK_a of pK_1.
[c] From Turnbull *et al.* (1992), in 0.1 M MES, 0.051 M *N*-ethylmorpholine, and 0.051 M diethanolamine with 0.2 mM EDTA and 2 mM β-mercaptoethanol.
[d] K_m^{app} value calculated from $k_{cat}/K_m^{app} = Y'_b$ for species H'_1X and/or XH'_2.

can assess the independence of the effects of assembly and the Y165F mutation: does $\Delta\Delta G$ ($c_3^{wt} \rightarrow c_6r_6^{wt}$) + $\Delta\Delta G$ ($c_3^{wt} \rightarrow c_3^{mut}$) = $\Delta\Delta G(c_3^{wt} \rightarrow c_6r_6^{mut})$? In other words, do the assembly and mutation coupling energies sum additively in the mutant holoenzyme? In Scheme III $\Delta\Delta G_{2' \rightarrow 2'}(c_3^{wt} \rightarrow c_6r_6^{wt})$ + $\Delta\Delta G_{2' \rightarrow 2'}(c_3^{wt} \rightarrow c_3^{mut})$ = 2.16 kcal/mol, while $\Delta\Delta G_{2' \rightarrow 3'}(c_3^{wt} \rightarrow c_6r_6^{mut})$ = 0.4 kcal/mol and $\Delta\Delta G_{2' \rightarrow 4'}(c_3^{wt} \rightarrow c_6r_6^{mut})$ = −0.71 kcal/mol, indicating that assembly and the Y165F mutation are not independent in their effects on the active site in the mutant holoenzyme.

In both Michaelis and binary complexes there are 32 possible linkages of the type shown above among the pK values in c_3^{wt}, c_3^{mut}, $c_6r_6^{wt}$, $c_6r_6^{mut}$. All but one of these 64 possible linkages show nonindependent effects of assembly and mutation, indicating that there is general long distance energetic coupling among residue Tyr-165, the subunit interfaces altered in $c_3 \rightarrow c_6r_6$ assembly, and the active site

Tyr-165
/ \
c:r assembly — active site

SCHEME IV.

such that changes in one of the three "pairwise" relationships lead to compensatory changes in the other two. It is unlikely that these three regions of the molecule represent an isolated three site coupling, however. More likely, these results reflect global long range coupling throughout the enzyme that can profoundly and interdependently influence the electrostatics at the active site.

The one exception to this global coupling is the linkage in the binary complex between pK'_1 in c_3^{wt} and the highest pK values in c_3^{mut}, $c_6r_6^{wt}$, and $c_6r_6^{mut}$. The finding of an independent set of pK_a values in the binary complex, but not in the Michaelis complex, may be indicative of the T to R conformational switch. Data from equilibrium isotope exchange kinetics indicate that the T to R transition accompanies the binary to Michaelis complex transition, possibly beginning just before or simultaneously with the binding of the first asparate. (Hsuanyu and Wedler, 1987). Residue Tyr-165 shifts from an interchain to an intrachain interaction when the enzyme switches from the T to the R state. This shift will change the way residue Tyr-165 is energetically coupled to the rest of the molecule and the observed loss of this one independent set of pK_a values may be a marker for the allosteric switch.

DISCUSSION

This study represents the first detailed analysis of the pH dependence of the kinetic parameters of both the holoenzyme and a set of single-site mutants. Two levels of questions are addressed: the effects of assembly on the catalytic mechanism of the holoenzyme and the effects of mutations.

Assembly and the Catalytic Mechanism

There are obvious qualitative differences in the pH dependence of the kinetic parameters of the holoenzyme and catalytic subunit. Whereas both log V_{max} and log V_{max}/K_m for c_3 have a bell-shaped dependence on pH (Leger and Hervé, 1988; Turnbull *et al.*, 1992), log V_{max} for the holoenzyme is a half-bell, and

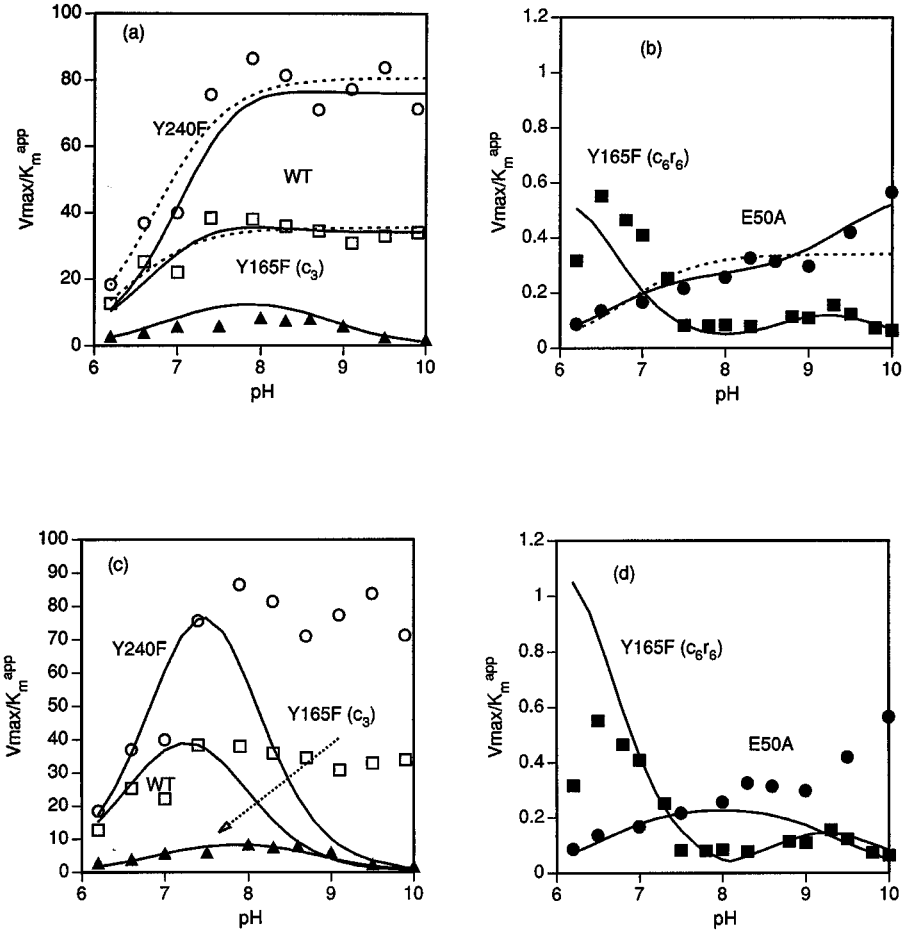


FIG. 6. **Calculated pH dependence of V_{max}/K_m^{app} of specific protonation species.** *Solid lines* are calculated V_{max}/K_m^{app} values from the species distributions and Y values for the fits to two pK_a values; *dashed lines* are calculated values from the fits to a single pK_a. Symbols are experimental values: WT (□), E50A (●), Y240F (○), Y165F c_6r_6 (■), Y165F c_3 (▲). The units are mmol/mg·h for V_{max} and mM for K_m^{app}. *a* and *b* demonstrate reasonable agreement between the experimental data for the wild-type enzyme and Y240F with both the one-group model in which X is the active species and the two-group model in which species XH'_2 and X contribute to productive binding. Only the second model fits the data for E50A. Also shown is the comparison for the four group model with two active species for Y165F. *c* and *d* show that the V_{max}/K_m^{app} values calculated from Y'_b and the distribution of XH'_1, the catalytically productive species in the model of Turnbull *et al.* (1992), do not agree with the experimental data for the wild-type enzyme, Y240F and E50A. The calculated values have been scaled to account for the fact that XH'_1 is a minor species generated by reverse protonation.

log V_{max}/K_m plateaus above pH 8. These differences raise the question of whether the catalytic mechanisms of the c_3 subunit and holoenzyme differ, or whether such differences result simply from modest quantitative shifts in pK values of the same functional groups.

Two models of the catalytic mechanism of the catalytic trimer have been proposed. In their analysis of the pH dependence of the catalytic trimer, Leger and Hervé (1988) suggested that groups with pK_a values of 7.1 and 9.1 in the binary complex of c_3 with carbamyl phosphate are involved in binding L-Asp and that a group with a pK of 7.3 is involved in catalysis. Turnbull *et al.* (1992) proposed that binding of L-Asp involves a group that must be protonated for binding with a pK of 7.3 in the binary complex with carbamyl phosphate and a p$K > 9$ in the ternary complex. Catalysis was proposed to involve two groups: one with a pK of 9.1 in the binary complex and 7.2 in the Michaelis complex that must be deprotonated and a second group with a pK of 9.5 in the Michaelis complex that must be protonated. A novel feature of this model is reverse protonation of the catalytically productive form of the binary complex with carbamyl phosphate. This in turn requires that the catalytically productive form be a minor species (<0.1%).

One possible explanation for the change in shape of the log V_{max}/K_m^{app} profile for c_6r_6 relative to c_3 is that L-Asp is a sticky substrate for c_6r_6, with a rate of dissociation less than k_{cat}. This seems unlikely as values of k_{cat} and K_m^{app} are similar for the holoenzyme and catalytic subunit, and the possibility that L-Asp is a sticky substrate for the catalytic subunit has been ruled out by Turnbull *et al.* (1992). Instead, the fits reported in Tables I and II and shown in Fig. 5 suggest that there are significant differences between the carbamyl phosphate bound binary complexes of c_6r_6 and c_3. This would not be surprising, since subunit interactions in the holoenzyme, particularly those between the catalytic subunits at the c1:c4 interface, constrain active site residues in the holoenzyme to a greater extent than they are constrained in c_3.

On the other hand, the log V_{max} plot for the holoenzyme can be fit with the same model used to fit c_3; the fitted pK values differ by ≤ 1 pH unit (7.9 *versus* 7.2; 10.5 *versus* 9.5; from Table I), and the turnover number and K_m^{app} of the putative active species are very similar (750 *versus* 680 s^{-1} and 16 *versus* 8 mM; from Table II). At the saturating substrate concentrations at which V_{max} is determined, domain closure in the holoenzyme creates a highly constrained active site in which the interactions of many side chains with the substrates are optimized. Since domain closure would be expected to be at least as efficient in c_3, this step in the catalytic mechanism might be expected to be the same in both proteins. The half-bell log V_{max} profile of c_6r_6 can be accounted for simply by the one pH unit increase in the pK of the more basic group involved in catalysis. Modeling indicates that a very small change in the relative positions of two charged side chains is sufficient to generate a pK change of this magnitude (Oberoi *et al.*, 1995).

Effects of Mutations

Mutation and Mechanism—Mutant E50A was originally designed to impede domain closure by eliminating interdomain salt bridges between Glu-50, Arg-167, and Arg-234 in the R state (Newton and Kantrowitz, 1990). It has been shown to have a random kinetic mechanism (Lee *et al.*, 1995). The similarities between Y165F and E50A may indicate that domain closure is also difficult in Y165F, and this may in turn give rise to a random kinetic mechanism, lack of substrate inhibition, and a shift in the pH optimum for V_{max}. However, a change in

kinetic mechanism (the order and relative rates of binding and catalytic events) does not imply a change in the catalytic mechanism (the specific residues involved in facilitating catalysis and their functions). It may be, however, that a change in kinetic mechanism from ordered to random substrate binding results in residues involved in the carbamyl phosphate portion of the active site influencing the apparent pH dependence of aspartate binding.

The T to R Transition—Since the mutated residues are located in regions of the holoenzyme whose three-dimensional structure changes during the T → R transition and the T → R transition and the ionization state of the protein are known to be linked (Gerhart and Pardee, 1964; Thiry and Hervé, 1978), the results presented here allow a number of long standing questions regarding aspartate transcarbamylase to be addressed.

The first is the question of whether pH alone can induce the T to R switch in some mutants. In principle, it would be possible for mutants that appear T-like at one pH to become R-like at a different pH, and *vice versa*. However, since the variations in substrate affinity and catalytic activity with pH of any individual mutant are much less than those produced by mutation, radical changes in quaternary structure with pH appear unlikely. Instead, the Hill coefficients of the mutants vary less with pH than the wild type, suggesting their ability to undergo the T → R transition is reduced, or that in some way the structural-energetic "distance" between the T and R states, which gives rise to cooperativity, is diminished in the mutant enzymes. This is turn bears on two other questions that have frequently been raised.

1) Can mutations "lock" the enzyme into the T or R state? If the mutant enzyme exhibits cooperativity, manifested either by having a Hill coefficient >1 in Equation 3 or by curvature in a Hanes plot (see also Allewell and LiCata (1995)), then it obviously cannot be locked in the T or R state. Many mutants are noncooperative under some conditions and cooperative under other conditions. Mutants that have previously believed to be locked into one conformation have later been found to be cooperative under other solution conditions; for example E50A of this study and E239Q.[4] This question can be answered in the affirmative only after an exhaustive examination of the behavior of the mutant.

2) Can pH dependences at high and low aspartate concentrations be used as a conformational probe of the enzyme (*i.e.* as a T *versus* R state indicator)? It has been known for many years that in wild-type aspartate transcarbamylase the pH dependence of velocity at very low [L-Asp] has a maximum at pH 7, while at high [L-Asp] the velocity maximum shifts to pH 8.3 (Gerhart and Pardee, 1964). These optima are generally assigned to the T and R states of the enzyme, respectively. The exact positions of these optima depend on solution conditions and on the specific aspartate concentrations used to determine them. Since K_m^{app}, V_{max}, and n_H for aspartate transcarbamylase all change with pH, the apparent velocity at a single [L-Asp] over a range of pH cannot be related to any single kinetic property of the enzyme. When full titration curves are collected as a function of pH, as in this study, it can be seen that there is not a one to one correlation between the pattern of pH dependence and the allosteric state of the enzyme. Both the V_{max} and the K_m^{app} of the wild-type enzyme and Y240F are maximal at about pH 9 (Fig. 3). These are opposing effects, *i.e.* catalysis is most rapid at pH 9 and slowest at pH 6–7, while binding is tightest at pH 6–7 and weakest at pH 9. For Y165F and E50A both the highest V_{max} and the weakest K_m^{app} occur near pH 7 (pH 7.5 for Y165F K_m^{app}). Examining the pH dependence in the usual way (*i.e.* at one low and one high [L-Asp]) will yield a composite of these two opposing effects that varies at each pH. Surely the weakest K_m is not associated with the R state of the enzyme, so is it reasonable to assume that the high pH maxima of wild-type and Y240F reflect an R state enzyme? Likewise, associating the pH 7 maxima of the low activity mutants with a T-like state would require that the highest k_{cat} be associated with that state. In addition, how can classifying E50A as either a T-like or R-like be rationalized with the nearly exact equivalence of the magnitude and pH dependence of n_H for E50A and the wild-type enzyme? It would appear then, that although a cursory examination makes this diagnostic seem quite attractive, without additional types of information, the position of the pH maxima for an aspartate transcarbamylase mutant should not be used as an indicator of the conformational state of the enzyme.

The question of whether mutations can push aspartate transcarbamylase into a stable intermediate state (*i.e.* different from T or R) is also frequently raised. The term "intermediate state" is quite broad and can be operationally defined in a number of ways. Structural methods are the best way to address the question of intermediates. Attempting to establish intermediate forms from functional studies and mutational analyses in a system where cooperativity is still characterized only by a Hill coefficient is inadvisable (see Ackers *et al.* (1992) for an example of the type of approach necessary).

Long Distance Electrostatic Interactions—The relationship between changes in residues distant from the active site and changes in the pH dependence of enzymatic activity has been a topic of interest to enzymologists and protein engineers for some time (*cf.* Thomas *et al.* (1985) and Russell and Fersht (1987)), as has been the challenge of predicting these interactions from electrostatic modeling (Gilson and Honig, 1987; Sternberg *et al.*, 1987). Analysis of the crude thermodynamic cycles linking holoenzyme assembly, enzyme activity, and residue Tyr-165 point toward long range energetic coupling networks within aspartate transcarbamylase that are manifested in the enzyme's pH dependence.

The mutations characterized in this study yield further information about the nature of long distance coupling in the molecule. The smallest distances between any atom of the bisubstrate analog PALA and any atom of Tyr-240 and Tyr-165 are 16 and 14 Å, respectively. If the intramolecular interactions that produce the observed pK_a shifts depended only upon distance, the effects of removing these tyrosines should be nearly identical, while the pK_a shift caused by altering Glu-50, which is much closer to the active site, should be larger than either of the other two substitutions. This is clearly not the case. The significant quantitative difference between the two nearly equidistant Tyr to Phe substitutions demonstrates specific path dependence of this coupling, as opposed to either distance dependence or a dependence on the type of substitution.

These functional studies allow us to observe pK_a changes at the active site upon mutating sites elsewhere in the protein. Electrostatic modelling allows us to look at the same interaction in the opposite direction. Recent calculations using a multigrid approach to solving the nonlinear Poisson-Boltzmann equation (Oberoi and Allewell, 1993) indicate that active site ligation induces a ΔpK_a of < 0.3 pH units for Tyr-240, but causes the pK_a values of both Glu-50 and Tyr-165 to shift by > 1.5 pH units. Hence, both experimental studies of the pH dependences of the functional parameters and electrostatic calculations predict a specific, strong interaction between the active site of the enzyme and residues Tyr-165 and Glu-50 and only a weak interaction with residue Tyr-240. These correla-

[4] Yuan and Allewell, unpublished results.

tions of experiment and calculation support the premise that the interactions between the sites of mutation and the active site are predominantly electrostatic and suggest that engineering the activity of enzymes as complex as aspartate transcarbamylase by altering specific long range interactions predicted by modeling may be an achievable goal.

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